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Positive and Negative Strand of Hepatitis C Virus RNA Sequences in Peripheral Blood Mononuclear Cells in Patients With Chronic Hepatitis C: No Correlation With Viral Genotypes 1b, 2a, and 2b

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Hepatitis C virus (HCV) has many genotypes which are closely associated with the severity of chronic hepatitis and the response to antiviral therapy. Although HCV is essentially hepatotropic, several lines of evidence suggest that this virus can infect peripheral blood mononuclear cells (PBMC) in most patients with chronic HCV infection. However, the methods used previously to detect negative-strand HCV RNA have been questioned, and the PBMC tropism of different HCV genotypes remains unknown. A stringent method was used to investigate the prevalence of positive- and negative-strand HCV RNA in the PBMC of 106 patients with chronic hepatitis C and to analyze the influence of HCV genotype on the tropism of PBMC. HCV type 1b was the predominant strain in the patients. Positive-strand RNA in PBMC was detected in 83 (78%) and 40% had negative-strand RNA. The demographic and clinical features were comparable among different patients grouped by the replication status of HCV in the plasma and PBMC samples. In addition, there was no significant difference of PBMC tropism between type 1b and non-1b HCV. In summary, HCV does indeed infect actively the PBMC of chronic hepatitis C patients and such infection is not correlated to the pathogenesis of liver cell damage. Moreover, the genotype is not associated specifically with PBMC tropism of HCV. ***J. Med. Virol. 52:270–274, 1997.*** © 1997 Wiley-Liss, Inc.

KEY WORDS: HCV; negative strand; peripheral blood mononuclear cells (PBMC); chronic hepatitis C

INTRODUCTION

With the cloning of viral genome and the subsequent development of serologic assays [Choo et al., 1989; Kuo et al., 1989], hepatitis C virus (HCV) is now the major etiologic agent of parenteral non-A, non-B hepatitis worldwide [Chen et al., 1990; Houghton et al., 1991; Kao et al., 1994b]. The virus is an enveloped, 50–60 nm virus with a single positive-strand RNA genome of about 9.4 kb [Houghton et al., 1991]. Several lines of evidence suggest that HCV has a similar genetic organization to the pesti- or flaviviruses [Houghton et al., 1991]. Comparative sequence analysis showed significant genetic heterogeneity among isolates from different geographic areas and even within isolates from a single individual [Martell et al., 1992; Bukh et al., 1995; Kao et al., 1995a]. Recent phylogenetic analysis of the NS5 region of the HCV genome has classified the virus into 6 major groups and 11 subtypes [Simmonds et al., 1993]. The HCV genotypes have been shown to be closely associated with the severity of chronic hepatitis and the response to antiviral therapy [Bukh et al., 1995; Kao et al., 1995b].

Much of the cellular tropism of HCV remains to be clarified. Although it is hepatotropic, previous studies have suggested that HCV can infect peripheral blood mononuclear cells (PBMC) in most (70–100%) patients with chronic hepatitis C by using so-called strand-specific reverse transcription-polymerase chain reaction (RT-PCR) to detect positive or negative-strand

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HCV RNA [Fong et al., 1991; Bouffard et al., 1992; Qian et al., 1992; Wang et al., 1992; Zignego et al., 1992; Gil et al., 1993; Muller et al., 1993; Muratori et al., 1994; Navas et al., 1994; Saleh et al., 1994; Kusaka et al., 1995; Ounanian et al., 1995; Taliani et al., 1995]. The presence of HCV in PBMC may have implications in viral transmission, persistence, pathogenesis, autoimmunity, reinfection of transplanted liver, and in the response to antiviral therapy [Oldstone, 1991; Feray et al., 1992; Lunel, 1994; Navas et al., 1994; Saleh et al., 1994; Kusaka et al., 1995; Ounanian et al., 1995; Taliani et al., 1995], and thus is important. However, the methods used previously for detection of negative-strand HCV RNA have been challenged recently because they lack sufficient strand specificity [Willems et al., 1993; Gunji et al., 1994; McGuinness et al., 1994; Lanford et al., 1995]. In addition, the PBMC tropism of different HCV genotypes remains unknown. We therefore employed a stringent strand-specific PCR method to investigate the prevalence of positive- and negative-strand HCV RNA in the PBMC of 106 patients with chronic hepatitis C. We examined the possible influence of HCV genotype on the tropism of PBMC and correlated the replication in PBMC with clinical characteristics.

MATERIALS AND METHODS

Patients

From July 1994 to June 1995, a total of 106 consecutive patients (49 men, 57 women; ages 22–83 years) with chronic hepatitis C and defined HCV genotypes from the gastroenterological clinic of National Taiwan University Hospital were enrolled. HCV infection was defined by positivity for serum anti-HCV (second-generation assay, Abbott Laboratories, North Chicago, IL) as well as HCV RNA in plasma and/or PBMC. They had elevated serum alanine aminotransferase (ALT) levels (upper limit of normal, 31 IU/L) for at least 1 year, all were negative for HBsAg in the serum, and they did not have markers of autoimmune hepatitis including antinuclear, antimitochondrial, and anti-smooth muscle antibodies. None had a history of renal dialysis, alcoholism (>50 g/day), intravenous drug use, homosexuality, or hepatotoxin exposure. Metabolic liver disease including Wilson's disease, hemochromatosis, or α-1 anti-trypsin deficiency was excluded by clinical and laboratory data.

Separation of PBMC and RNA Extraction

PBMC were isolated from 20 mL of whole blood mixed with sodium citrate by centrifugation over a Ficoll-Hypaque density gradient and washed three times in 10 mL of phosphate-buffered saline (PBS). Aliquots of paired plasma and 1×10^6 PBMC samples were stored at −70°C until use. Total RNA was extracted from 100 μL of plasma, 500 μL of final PBMC washing, and 1×10^6 unthawed PBMC by the single-step acid guanidinium thiocyanate-phenol-chloroform method.

TABLE I. Nucleotide Sequences of the Genotyping Primers

Primer no.[a]	Sequence (5′ → 3′)	Nucleotide position
C3s	CGAAAGGCCTTGTGGTACTG	−70–−51
186a	ATGTACCCCATGAGGTCGGC	410–391
104s	AGGAAGACTTCCGAGCGGTC	148–167
132a	TGCCTTGGGGATAGGCTGAC	204–185
133a	GAGCCATCCTGCCCACCCCA	291–272
134a	CCAAGAGGGACGGGAACCTC	321–302
135a	ACCCTCGTTTCCGTACAGAG	270–251

[a]s = sense; a = antisense.

Determination of HCV Genotypes and Specific Amplification of Positive- and Negative-Strand HCV RNA

HCV RNA and genotypes were identified by Okamoto's type-specific primers with slight modification of the primer sequences (Table I). The nomenclature was based on a widely accepted system [Simmonds et al., 1993]. The presence of positive- and negative-strand HCV RNA in plasma and PBMC samples was detected by strand-specific RT-PCR. The anti-sense (186a) and sense (C3s) outer primers were used to reverse transcribe the positive and negative strands of HCV RNA, respectively. The cDNA was amplified subsequently by nested PCR with outer primer pairs for the first stage and the inner primer pairs for the second stage to identify the HCV genotypes. Plasma samples from patients with defined types 1b, 2a, or 2b virus infection were mixed and used as positive controls, while samples from healthy persons and reagents without DNA were used as negative controls. The sensitivity and specificity of this typing assay have been demonstrated [Kao et al., 1994a]. To avoid detection of "false"-negative strand, the following stringent method was used after cDNA synthesis [McGuinness et al., 1994]: heat treatment at 95°C for 2 hours to inactivate reverse transcriptase activity and RNase H (1 unit, Promega) at 37°C for 30 minutes to digest total RNA to prevent possible RT and subsequent amplification of positive RNA strands by Taq polymerase. The specificity of the detection of negative-strand HCV RNA in both plasma and PBMC was confirmed by the absence of nonspecific amplification when the synthesis of cDNA without adding reverse transcriptase for exclusion of PCR product contamination, and a PCR assay using all components except the sense primer in the transcription step to assess total reverse transcriptase inactivation and RNA degradation by heat and RNase H, respectively.

To avoid false positive results by contamination, the prevention measures of Kwok and Higuchi [1989] were followed strictly, and negative controls were included in all experiments.

Statistical Analysis

Group comparisons were analyzed by Chi-square test with Yates' correction and analysis of variance where appropriate. A P value of <0.05 was considered statistically significant.

TABLE II. Definition of Groups by HCV Status in Plasma and PBMC

Group	No. of cases	Plasma: Positive-/negative-strand	PBMC: Positive-/negative-strand
A	27	+/–	+/+
B	39	+/–	+/–
C	23	+/–	–/–
D	6	–/–	+/+
E	11	–/–	+/–

RESULTS

HCV type 1b was identified in 71 of 106 samples (67%), type 2a in 22 (21%), type 2b in 10 (9%), and mixed infections in 3 (3%). HCV RNA was not found in the final washing samples of PBMC. Positive-strand HCV RNA in PBMC was detected in 83 (78%), and 33 (40%) had negative-strand HCV RNA. Based on positive- and negative-strand HCV in the plasma and PBMC samples, these patients were divided into five groups (A, B, C, D, and E in Table II). Thirty-three patients had negative-strand HCV RNA in PBMC, but none had negative-strand HCV RNA in the plasma.

The demographic and clinical features with respect to sex, mean age, history of blood transfusion, and peak serum ALT level were comparable in the five groups of patients (Table III). The distribution of HCV genotypes in the five groups is shown in Table IV, and there existed no significant difference of PBMC tropism between genotype 1b and other genotypes of HCV (Table V).

DISCUSSION

Although the nucleotide sequences and genomic organization of several HCV isolates have been determined, the exact mechanism of viral replication is far from clear because of the lack of a suitable culture system. However, replication of flaviviruses has been shown to involve the synthesis of complementary genomic-length negative-strand RNA by using the positive strand as a template [Chambers et al., 1990]. Conversely, positive-strand RNA is then amplified by using the negative strand as a template. Thus, detection of negative-strand RNA in infected cells is considered a marker of active viral replication. Recently, several studies described strand-specific RT-PCR methods for selectively detecting positive- and negative-strand HCV RNA in serum, PBMC, or liver tissue from a limited number of patients with chronic hepatitis C [Fong et al., 1991; Bouffard et al., 1992; Qian et al., 1992; Wang et al., 1992; Zignego et al., 1992; Gil et al., 1993; Muller et al., 1993; Muratori et al., 1994; Navas et al., 1994; Saleh et al., 1994; Kusaka et al., 1995; Ounanian et al., 1995; Taliani et al., 1995]. The results showed that infection and replication of HCV can occur in PBMC of most patients. However, the strand specificity and/or intracellular localization of HCV sequences detected in cell samples by heat inactivation of reverse transcriptase alone has been questioned [Willems et al., 1993; Gunji et al., 1994; McGuinness et al., 1994; Lanford et al., 1995]. Because cDNA can still be synthesized in the presence of Taq polymerase (RT activity in vitro), that may lead to false positive results. Accordingly, several investigators treated the samples with RNase after heat inactivation to eliminate both positive and negative strands of RNA to further minimize the possible false positivity [Willems et al., 1994; Zignego et al., 1995].

By using a similarly stringent method, we studied the prevalence of HCV infection of PBMC in a larger number of patients with chronic hepatitis C. The fact that none of the plasma samples was positive for negative-strand HCV RNA confirmed the strand specificity of our present method. In agreement with previous reports [Willems et al., 1994; Zignego et al., 1995], our results showed that positive-strand HCV RNA in PBMC was detected in 78% of 106 chronic hepatitis C patients and 40% had both strands of RNA in the PBMC samples. These data suggested that HCV indeed infects human PBMC and such cells can be a site for extrahepatic viral replication.

There is still a proportion of chronic hepatitis C patients whose PBMCs are not infected by HCV. Although the mechanisms involved in PBMC tropism of HCV remain unclear, recent studies on human immunodeficiency virus type 1 (HIV-1) indicated that the antigenic variation in the hypervariable V3 loop sequence of envelope glycoprotein (gp120) contributes to lymphocyte tropism [Hwang et al., 1991; Chesebro et al., 1992; Keys et al., 1993; McKnight et al., 1995]. Thus, further studies are required to determine whether specific sequences exist in the hypervariable region (HVR-1) of the HCV genome for PBMC tropism as is demonstrated in the V3 loop of HIV-1.

Detection of HCV RNA in PBMC, but not in plasma of a given patient, has been reported previously [Muratori et al., 1994; Navas et al., 1994; Kusaka et al., 1995; Taliani et al., 1995]. Also, Shimizu et al. [1992] demonstrated recently that certain human lymphocytic cell lines could be infected with HCV, but viral sequences were detected sporadically in the supernatants of the cultures postinoculation. We also found 17 such patients (groups D and E) in the present study. Although the mechanism involved is not yet known, the presence of negative-strand HCV RNA in PBMC, but not in plasma samples, argues strongly against simple adherence of HCV to the PBMC membrane. It may be reasoned that the replication of HCV in PBMC does not appear to be as robust as that in liver cells; that HCV-infected PBMC may not be able to form and release the hepatitis C virions into blood circulation, or that the level of plasma HCV RNA released from PBMC may be too low to be detected.

The correlation between the presence of HCV RNA in PBMC and the clinicopathologic course of chronic hepatitis C remains unclear. Our data showed that the demographic and clinical features, including peak serum ALT level, were comparable among different patient groups (Table III). This indicates that PBMC infection

TABLE III. Clinical Characteristics of Patients With Chronic Hepatitis C in Different Groups

Characteristics	Group[a] (No.) A (27)	B (39)	C (23)	D (6)	E (11)	*P* value
Sex (M/F)	13/14	18/21	12/11	3/3	3/8	NS
Age (years)	56 ± 10	56 ± 12	58 ± 12	54 ± 10	57 ± 15	NS
Transfusion history	7 (26%)	13 (33%)	6 (26%)	1 (17%)	2 (18%)	NS
Peak ALT (IU/L)	94 ± 66	129 ± 137	108 ± 94	104 ± 69	157 ± 160	NS

[a]A = plasma (+/–), PBMC (+/+); B = plasma (+/–), PBMC (+/–); C = plasma (+/–), PBMC (–/–); D = plasma (–/–), PBMC (+/+); E = plasma (–/–), PBMC (+/–). The definition of each group is shown in Table II.

TABLE IV. Prevalence of HCV Genotypes in Different Groups

Group[a] (No.)	Genotype 1b No. (%)	2a No. (%)	2b No. (%)	Mixed No. (%)
A (27)	21 (78)	3 (11)	1 (4)	2 (7)
B (39)	24 (61)	10 (26)	5 (13)	0 (0)
C (23)	14 (61)	4 (17)	4 (17)	1 (5)
D (6)	5 (83)	1 (17)	0 (0)	0 (0)
E (11)	7 (64)	4 (36)	0 (0)	0 (0)
Total (106)	71 (67)	22 (21)	10 (9)	3 (3)

[a]A = plasma (+/–), PBMC (+/+); B = plasma (+/–), PBMC (+/–); C = plasma (+/–), PBMC (–/–); D = plasma (–/–), PBMC (+/+); E = plasma (–/–), PBMC (+/–). The definition of each group is shown in Table II.

TABLE V. Influence of HCV Genotypes on Tropism of PBMC

Genotype (no.)	PBMC positive for HCV RNA No. of patients (%)	*P* value*
1b (71)	57 (80)	0.8
Non-1b (32)	24 (75)	

*Chi-square test with Yates' correction.

can be present at any stage of the natural course of chronic hepatitis C and may not contribute to the pathogenesis of liver cell injury. In addition, although HCV type 1b has been shown to be closely associated with the progression of chronic hepatitis and poor response to antiviral therapy [Bukh et al., 1995; Kao et al., 1995b], we found similar distributions of HCV genotypes in five groups of patients. Also, no significant difference of PBMC tropism between type 1b and non-1b HCV was found, suggesting that HCV genotypes had no influence on the tropism of PBMC.

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